*ACETYLCHOLINESTERASE, I. LARGE-SCALE PURIFICATION, HOMOGENEITY, AND AMINO ACID ANALYSIS**

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According to theory, the action of acetylcholine (ACh) initiates a series of reactions which are responsible for the increased permeability to ions of excitable membranes during electrical activity; acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) rapidly catalyzes the hydrolysis of the ester, thereby permitting the membrane permeability to return to its resting state.[1, 2] In view of this paramount role of acetylcholinesterase (ACh-esterase) in the elementary process of such a vital function as the conduction of nerve impulses in nerve and muscle fibers, this enzyme has been intensively studied during the last two decades in many laboratories all over the world. Much information has accumulated through kinetic studies about the mechanism of reactions with substrates and inhibitors, and about the molecular forces in its active site (e.g., see refs. 3–6). These analyses led to the understanding of the mode of action of many important drugs acting on the nervous system and of the effects of potent and toxic compounds such as, for instance, the organophosphates, to which belong the nerve gases and insecticides.

In contrast, in spite of the rapid advances of protein and macromolecular chemistry in general during the last 15 years, and the increasing insight into the crucial role of macromolecules in cellular functions, very little information is available about the protein properties of ACh-esterase. The main obstacle has been that pure enzyme protein has not been available until now in adequate amounts. The work presented here reports a convenient method that permits, for the first time, a yield of large amounts of pure enzyme adequate for analyzing the properties of the enzyme protein.

A highly active solution of ACh-esterase was first obtained by Nachmansohn and Lederer,[7, 8] in 1939, from electric tissue of *Torpedo marmorata*. The extraordinary value of this material for isolating the enzyme became apparent when it was recognized that 1 kg of the tissue, with a protein content of only about 30 gm, metabolizes 2–4 kg of ACh per hour. Several hundredfold purification of the enzyme solution extracted from the electric tissue of *Electrophorus electricus*, which is comparable to that of *Torpedo*, was obtained in the 1940's with a specific activity of 440 mmoles of ACh split per mg protein per hour.[9] In a few preliminary observations with analytical ultracentrifugation of this preparation, a rather high sedimentation rate was observed, with a major peak suggesting a large molecular weight of more than one million; however, the polydispersed nature of the material did not support the assumption of a homogeneous protein.

Attempts at purification, discontinued due to difficulties of procuring adequate amounts of material, were again taken up in the late 1950's by Lawler.[10] Using some improved but basically similar methods, she obtained the same degree of specific activity. She estimated the molecular weight, on the basis of active centers, to be 240,000. An interesting but unexplained observation was that of

an extraordinarily high polymer, obtained in a few preparations, with a rather sharp single peak and a sedimentation coefficient of 60–80, depending on the concentration of the protein.[11] This corresponds to a molecular weight of about 13×10^6, i.e., a polymer formed by about 50 units.

In the last decade many new procedures of enzyme purification have been developed. Using column chromatography, a 50 per cent higher specific activity was obtained than that originally observed: 660 mmoles/mg protein/hour.[12] A molecular weight of 230,000 was estimated in good agreement with that of Lawler. The reported procedures of exclusive column chromatography are not readily reproducible and proved to be unsuitable for large-scale preparation. Therefore, a revised procedure was developed for large-scale work and has proved reproducible.

Extraction and Purification Procedures.—Step 1: Fresh electric tissue from the main organ of *Electrophorus electricus* is cut in small pieces and stored under toluene at 2° (ref. 9). During a period of at least 8 weeks the tissue is periodically pressed with cheesecloth for the removal of the toluene and tissue exudate and resuspended in fresh toluene. All subsequent operations are conducted at 2–4°. Toluene-treated electric tissue in 1.0-kg lots is homogenized for 4 min in 2.5 liters of 5% (w/v) ammonium sulfate solution. The homogenized suspension is centrifuged for 1 hr at 10,000 × *g*, and the supernatant decanted through cheesecloth. The precipitate is rehomogenized with 0.8 liter ammonium sulfate solution per original kg of tissue and centrifuged. The supernatant is combined with the original extract, and the residue is discarded.

Step 2: To the combined extracts is added an equal volume of 0.01 *M* sodium phosphate, pH 7.0, containing sufficient ammonium sulfate to bring the final solution to 15% (w/v). Standard Super-Cel is added and the suspension filtered. To the clear filtrate, solid ammonium sulfate is added to bring the solution to 35% (w/v). Filter aid is added and the suspension is filtered to obtain a clear solution. The filter cake is extracted with 400 ml of 0.01 *M* sodium phosphate, pH 7.0, per kg of original tissue, the Super-Cel removed by filtration, and the clear solution dialyzed against 0.01 *M* sodium phosphate, pH 7.0, until free of sulfate.

Step 3: The clarified solution is adsorbed to a column of benzyl-diethylaminoethyl (B-DEAE) cellulose[11] equilibrated in 0.01 *M* sodium phosphate, pH 7.0, and with a bed volume of approximately 200 ml/kg of original tissue. Elution is accomplished with a linear gradient of buffered sodium chloride from 0 to 1.0 *M*. The fractions with high activity are combined and dialyzed until salt-free against deionized water, and lyophilized.

Step 4: The protein is redissolved in 0.01 *M* sodium phosphate, pH 7.0, at a concentration of approximately 50 mg/ml. After clarification at 45,000 × *g* for 30 min, the solution is applied to a Sephadex G-200 column previously equilibrated in the buffer and of the same bed volume employed for the preceding step with B-DEAE cellulose. The fractions with high activity are combined after elution with the 0.01 *M* phosphate buffer and adjusted to 0.03 *M* sodium phosphate, pH 5.9.

Step 5: The solution is next applied to a column of cellulose-phosphate, equilibrated under the same buffer conditions. After washing the column with a volume of 0.03 *M* sodium phosphate, pH 5.9, equivalent to the sample volume, elution is accomplished with buffered sodium chloride from 0 to 1.0 *M*.

Steps 6 and 7: The fractions from the cellulose-phosphate are neutralized, and the tubes with high activity are combined for subsequent anion exchange chromatography. The fractions are dialyzed against 0.03 *M* phosphate buffer, pH 7, before being applied to a column of DEAE-Sephadex equilibrated in the same buffer. Elution is accomplished with a linear gradient of buffered NaCl solution from 0 to 1.0 *M*. The top fractions are combined and the same step is then repeated with DEAE cellulose.

Assay methods: The enzyme activity in the earlier steps is determined by Hestrin's method.[13] For determining the activity of the purest fractions, a Radiometer automatic titrator was used.

The protein concentrations have been measured by their optical absorptions at 260 and 280 mμ[14] and at 215 and 225 mμ.[15] For continuous monitoring at 253.7 mμ of the effluents from chromatographic columns, the ultraviolet absorptiometer from LKB Instruments, Inc., has been used. For the exact protein values the nitrogen has been determined by Micro-Kjeldahl analysis.

Disk electrophoresis has been carried out with a Canalco disk gel electrophoresis apparatus under standard gel conditions. The homogeneity of the protein in size was tested by high-speed centrifugation with a Spinco model E ultracentrifuge and analyzed with schlieren optical system at 4°; the protein concentrations were 5–7 mg/ml. The amino acid analysis has been performed with a Beckman amino acid analyzer.

Results.—Table 1 summarizes the procedures used and the results obtained. The highest specific activity obtained was 750 mmoles/mg/hr. This is about 10–12 per cent higher than the best preparations described previously. In view of the extraordinarily high activity of the enzyme and the dilutions required for determining specific activity, a really precise estimate is difficult. The lowest figures have been used for the values of specific activity given. Therefore, the actual absolute figure may well be a few per cent higher, but this does not appear pertinent. With 10 kg toluene pretreated material, about 60 mg of homogeneous enzyme protein is obtained. The purity of this protein has been demonstrated with disk electrophoresis, as seen in Figure 1. For comparison two earlier steps are included in the figure.

The homogeneity of the protein is also apparent from the studies with high-

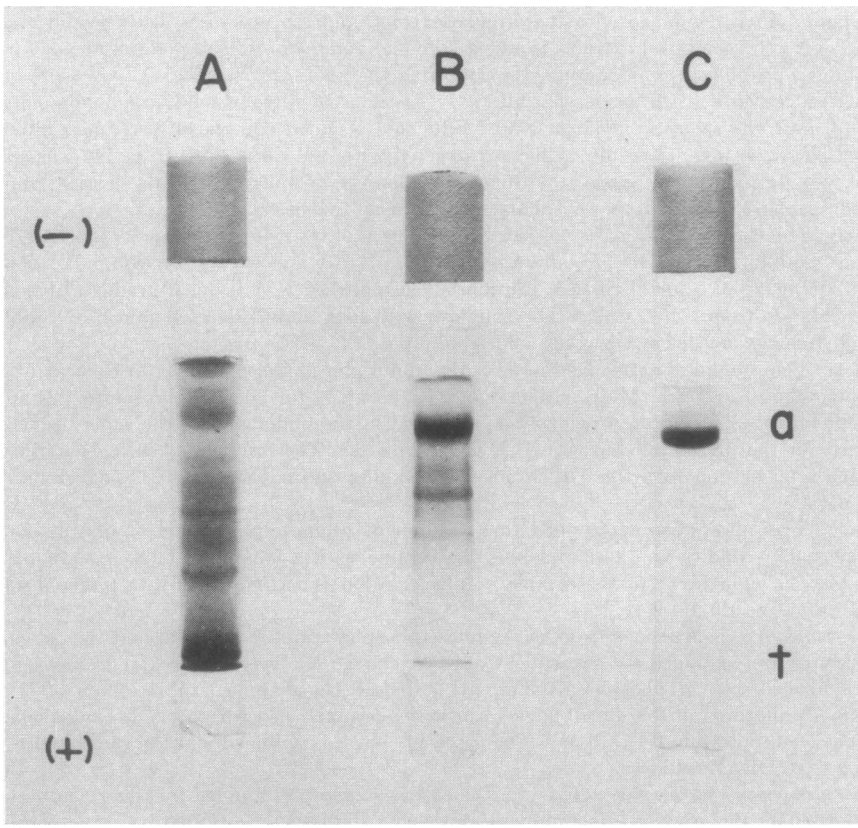

FIG. 1.—Results of disk electrophoresis. Gel patterns of ACh-esterase at three stages of purification: (*A*) 200 μg of protein with a specific activity (sp. act.) of 11; (*B*) same amount of protein, but with sp. act. of 100; (*C*) 20 μg of protein with sp. act. of 740. *t*, Tracking band, *a*, ACh-esterase band.

TABLE 1

SUMMARY OF THE PURIFICATION PROCEDURES AND RESULTS OBTAINED

Step	Procedure	Spec. activity	Total units	Yield (%)
1	Extraction and $(NH_4)_2SO_4$ precipitate	11–20	380,000–400,000	100
2	B-DEAE-cellulose	22–50	250,000–300,000	66–75
3	Dialysis	40–60	220,000–250,000	57–62
4	Sephadex	85–200	160,000–220,000	42–55
5	Cellex-P	230–350	110,000–150,000	29–37
6	DEAE-Sephadex	500–600	70,000–100,000	18–25
7	DEAE-cellulose	720–750	28,000– 45,000	8–12

Specific activity indicates mmoles of ACh hydrolyzed per mg protein per hour. The total enzyme activity referred to the total amount of protein present is given as total units.

speed centrifugation. With our preparation having a specific activity of 740 mmoles/mg/hr, a sharp peak has been obtained (see Fig. 2). In contrast to previous observations in which homogeneity in ultracentrifugation was observed with preparations of much lower specific activity (about 350 mM/mg/hr[16]), our preparation, even with specific activity of 570, still showed the presence of a contaminant. We explain this discrepancy by the much higher protein concentration used in our analyses, 5–7 mg/ml, as compared with 1–2 mg/ml in the previous report. This explains also why in the preceding studies a preparation with a specific activity of 660, showing only one peak in the ultracentrifuge, was not yet pure chromatographically.

The ultraviolet-absorption spectrum of the enzyme shows a minimum at 250 mμ and a maximum at 280 mμ; absorption above 320 mμ was negligible.

The molecular weight of the enzyme has been estimated, on the basis of gel filtration, to be approximately 250,000. This is in good agreement with previous estimates obtained on different basis by Lawler[10] and Kremzner and Wilson.[16] No effort has been made to obtain a more precise evaluation of the molecular weight, since it appears likely, in view of recent information on molecular size and multiple forms of enzymes, that this large molecule is a polymer formed by subunits. Studies are in progress to investigate this possibility.

An amino acid analysis has been performed. It provides valuable preliminary information for studies initiated and aimed at elucidating primary, secondary, and tertiary structures, conformation analyzed by optical rotatory dispersion and circular dichroism, absorption spectra, and the interpretation of titration curves. Table 2 summarizes the data of two different amino acid analyses carried out on two different preparations. In the values given in the table, the μmoles of amino acid residues are reported on the assumption of four histidine residues per minimum

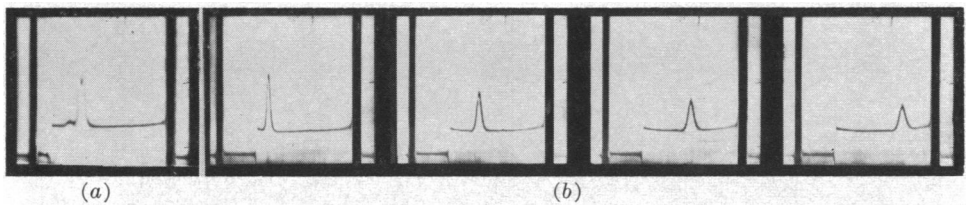

FIG. 2.—Sedimentation patterns of ACh-esterase with ultracentrifugation. (*a*) Preparation with sp. act. of 570 mM/mg/hr in phosphate buffer pH 7.0; the protein concentration was 7 mg/ml. The picture was taken 30 min after top speed was reached (59,780 rpm) (*b*) Preparation with sp. act. of 740; the protein concentration was 5 mg/ml, same buffer and speed. The pictures were taken 16, 32, 48 and 64 min after top speed was reached.

TABLE 2

AMINO ACID ANALYSIS OF ACETYLCHOLINESTERASE

	I*	II*		I*	II*
Lysine	8 (0.210)	8 (0.186)	Alanine	10 (0.2658)	10 (0.235)
Histidine	4 (0.1082)	4 (0.0984)	Half cystine	2 (0.053)	2 (0.0475)
Arginine	10 (0.255)	10 (0.239)	Valine	12 (0.338)	12 (0.295)
Tryptophan†	4 (0.100)	4 (0.090)	Methionine	5 (0.141)	5 (0.133)
Aspartic acid	20 (0.455)	21 (0.528)	Isoleucine	6 (0.178)	6 (0.159)
Threonine	8 (0.211)	8 (0.183)	Leucine	16 (0.432)	16 (0.386)
Serine	12 (0.334)	12 (0.293)	Tyrosine	7 (0.185)	7 (0.168)
Glutamic acid	16 (0.451)	16 (0.405)	Phenylalanine	10 (0.250)	10 (0.235)
Proline	14 (0.395)	14 (0.345)	Hexosamine	3 (0.078)	5 (0.118)
Glycine	14 (0.379)	13 (0.328)	Ammonia	20 (0.554)	18 (0.450)

The data have been obtained with two different preparations of the enzyme, both having a specific activity of 730 mM/mg/hr. The first preparation was the final result of the purification as described in Table 1. The second preparation was, in addition, submitted to a treatment with a hydroxyl apatite column. The actual analytical data (μmoles per sample) are presented in parentheses. The integers of amino acid residues are based on the assumption of four histidine residues per minimum molecular weight.

* Protein hydrolyzed in 6 *N* HCl, I, for 24 hr; II, for 22 hr.

† Tryptophan has been determined spectrophotometrically.[17]

molecular weight. The good agreement of the amino acid composition in the two different samples is considered, in conjunction with the other evidence, to be further support for the homogeneity of the protein preparation with a specific activity of 730.

Amino sugars were present in both analyses; however, there were three residues in the first, five in the second. The time of hydrolysis prior to the amino acid analysis was 24 hours in the first sample and 22 hours in the second. Hence, there was a slightly higher yield of ammonium in the first; the sum of ammonium and hexosamine is constant. For identification of the hexosamine part, a third analysis has been done with a hydrolysate performed with 4 *N* hydrochloric acid during 12 hours. The yield of amino sugars is slightly higher than in the second analysis. The elution curve shows three different peaks which are correspondent with glucosamine, galactosamine, and probably talosamine.

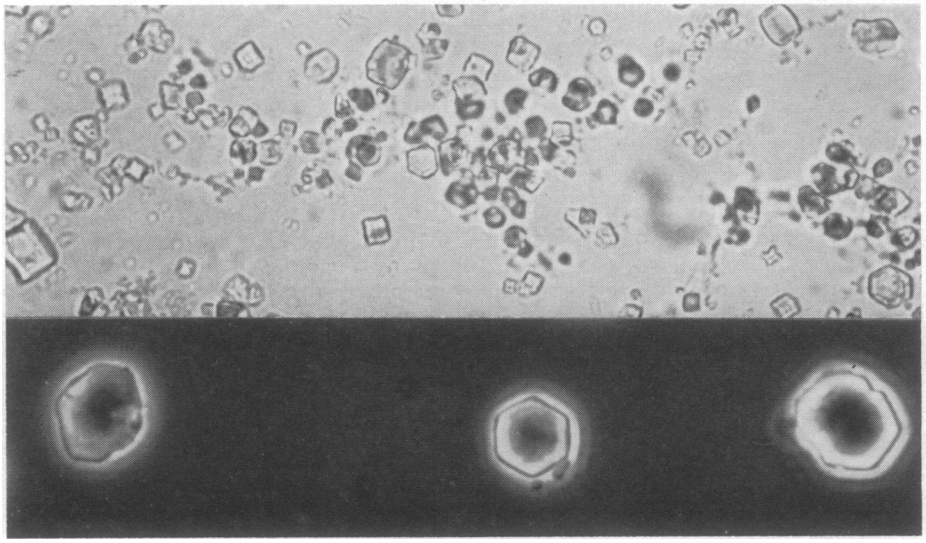

FIG. 3.—The upper part shows the crystals in the light microscope, × 600; the lower part presents a picture taken by Dr. A. Karlin with a biphase microscope, × 1250.

Summary.—The progress achieved, with the procedures described for purification of ACh-esterase of electric tissue of *Electrophorus electricus*, is the development of a method readily reproducible and suitable for a large-scale preparation. The preparation obtained appears to be a homogeneous protein, as tested by disk electrophoresis and high-speed centrifugation. Starting with 10 kg of toluene-treated material, about 60 mg of homogeneous enzyme protein is obtained. Thus, for the first time, studies of the protein properties of this biologically important enzyme have become possible. An analysis of this macromolecule appears pertinent for the understanding of its function in the elementary processes of excitable membranes during their activity.

Note added in proof: Shortly after the paper was submitted, crystals of the enzyme have been obtained in a 35% ammonium sulfate solution, which had been kept for some weeks in the refrigerator. The crystals have been recrystallized; they are hexagonal rods (Fig. 3). When a solution of these crystals was tested with disk electrophoresis, a single band was obtained. A detailed description will soon be presented.

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